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13. ABSTRACT (Maximum 200 Words) Matrix Metalloproteinases (MMP) are a family of enzymes that collectively degrade components of the extracellular matrix (ECM). A family of proteins called TIMP (Tissue Inhibitor of Metalloproteinase) are considered the primary protein inhibitors that modulate MMP activity. However, other polypeptides have been shown to inhibit MMP in vitro. MMP have been implicated in tumor growth and metastasis in general and breast tumors in specific. In the breast, MMP may also be involved in tumor fibrosis because the synthesis and degradation of ECM is no longer in balance. Understanding factors that modulate MMP activity is important to understand breast tumor biology. Previously, we identified a non-TIMP inhibitor that was the C-terminal region of Procollagen C-terminal Proteinase Enhancer. This novel inhibitor was designated CT-PCPE. Intact PCPE has no metalloproteinase inhibitor activity. Activity is revealed by proteolytic processing of the parent PCPE. We have observed CT-PCPE and other small non-TIMP inhibitors in the medium conditioned by an aggressive breast tumor cell line. To investigate the role of CT-PCPE in breast tumors, PCPE and CT-PCPE have been expressed for the purpose of characterization of inhibitor activity and CT-PCPE production. Additionally, another putative non-TIMP inhibitor has been identified in breast tumor cell conditioned medium.						
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Introduction

Many molecular mechanisms of tumor cell invasion and fibrosis in breast cancer are poorly understood. However, it is widely accepted for tumors to grow and invade, proteins of the extracellular matrix (ECM) must be degraded and remodeled. Several different classes of proteinases are involved in this process including matrix metalloproteinases (MMP). Except for a few exceptions, MMPs are activated in the extracellular space (Sternlicht and Werb, 2001). Once activated MMPs are inhibited by a family of protein inhibitors called Tissue Inhibitor of Metalloproteinases (TIMP) (Gomez *et al.*, 1997). In contrast to tumor invasion, breast tumor fibrosis involves the accumulation of ECM proteins such as collagen. For this to occur, the balance of deposition and turnover must be altered. This can be accomplished by an increase in the deposition of ECM proteins accompanied by inhibition of ECM degrading proteinases. For collagen to be deposited, a number of proteolytic processing steps are required. Nascent collagen must undergo N-terminal and C-terminal processing mediated by a specific procollagen N-terminal proteinase and a procollagen C-terminal proteinase (PCP) (Prockop *et al.*, 1998). A protein called Procollagen C-terminal Proteinase Enhancer (PCPE) enhances the activity of PCP. Enhancer activity resides completely within the N-terminal domain of the PCPE molecule (Takahara *et al.*, 1994). Previously, we identified a metalloproteinase inhibitory activity associated with the C-terminal domain of PCPE (CT-PCPE) (Mott *et al.*, 2000). Interestingly, the N-terminal metalloproteinase inhibitory domain of TIMP and the C-terminal domain of PCPE (i.e. CT-PCPE) share homology that may explain the observation that CT-PCPE can inhibit metalloproteinases (Trexler *et al.*, 2001; Banyai and Patthy, 1999). This activity was originally associated with fatal brain tumors; however, we have also observed this inhibitor in a highly invasive breast tumor cell line. This novel activity is revealed only when the parent PCPE molecule is proteolytically processed. It is possible that PCPE acts as a bifunctional molecule where the N-terminal domain enhances PCP activity while the C-terminal domain inhibits MMPs that could degrade nascent collagen before it is properly deposited and cross-linked in the ECM. Either function could contribute to the accumulation of ECM proteins seen in fibrosis. More importantly, MMPs and TIMPs have been traditionally investigated using cells derived from the stroma. However, breast tumors generally arise from epithelial cells. We have only observed these novel metalloproteinase inhibitors in epithelial cells. This suggests that there may be mechanisms in addition to TIMP that control metalloproteinase activity in cells of non-stromal origin.

The objectives of the proposed studies are to identify structural requirements for CT-PCPE to function as a metalloproteinase inhibitor; to determine mechanisms that generate CT-PCPE from PCPE; and to determine if increased expression of PCPE and/or CT-PCPE correlates with the invasive nature of breast tumor cells.

Body

Task 1 Identification of Functional Domain of CT-PCPE: Previous studies investigating metalloproteinase inhibitors in brain tumor conditioned medium revealed that in addition to the presence of TIMP a fourth activity was observed (Apodaca *et al.*, 1990). This activity was purified and identified by amino acid sequence analysis. Results indicated the polypeptide responsible for the inhibitor activity was the C-terminal domain of a protein previously identified as PCPE. Partial characterization of the inhibitory activity of CT-PCPE

against MMP has been examined (Mott et al., 2000). More recently, we observed CT-PCPE in the medium conditioned by an aggressive breast tumor cell line, MDA MB-231. CT-PCPE was identified in the breast tumor conditioned medium by inhibitory activity on reverse zymography and by western blot analysis with anti-PCPE antibodies. However, the inhibitors observed in the breast tumor conditioned medium differed slightly from the brain tumor inhibitor. Several inhibitor bands were observed at smaller molecular weights than CT-PCPE, and these bands did not react with the anti-PCPE. Two possible explanations for these observations were that the smaller bands were fragments of PCPE that did not react with the antibody, or these polypeptide inhibitors were not derived from PCPE.

To investigate the functional domain of CT-PCPE and to determine the identity of the inhibitor bands that did not react with the PCPE antibodies, several approaches have been undertaken. First, we attempted to identify the inhibitor fragments by directly sequencing the protein corresponding to these inhibitor activities from medium conditioned by MDA MB 231 cells. In doing this, we have uncovered a potential new inhibitor, a fragment of a protein known as Arginine Rich Protein (ARP). Mutations within this gene have been found in patients with a variety of cancers, including breast cancer (Shridhar et al., 1996a; Shridhar et al., 1996b) It is unclear what role if any ARP plays in cancer progression (Evron et al., 1997). However, one of the most intriguing observations made from the comparison of the amino acid sequence of CT-PCPE, the inhibitory domain of TIMP, and the ARP fragment is the number and placement of cysteine residues, which are similar in these three polypeptides. The metalloproteinase activity of TIMP and CT-PCPE are dependent on intact and correctly paired cysteines (Mott et al., 2000; Caterina et al., 1997; Gomez et al., 1997). We are currently investigating the possibility that ARP represents another non-TIMP metalloproteinase that is expressed in epithelial cells.

A second approach used to determine the functional unit of CT-PCPE was to express different lengths of CT-PCPE polypeptide in a bacterial expression system. This was done in collaboration with Dr. Richard Williamson, University of Kent, Canterbury, U.K. After expression, the polypeptides were refolded using conditions previously established by Dr. Williamson's laboratory for the refolding of TIMP expressed in bacteria. We have tried four different lengths of CT-PCPE. Unfortunately, we have been unable to detect activity with these expressed polypeptides. Many possible reasons may exist for this result including improper folding from the bacterial system even though the cysteine residues appear to be paired correctly. Because these studies with bacterially expressed CT-PCPE were not fruitful, an alternative strategy has been employed. Full length PCPE was expressed in the mammalian cell line HEK-293. A mammalian expression system was utilized in order to minimize any folding or glycosylation issues. An expression vector that allowed secretion of the protein into the conditioned medium was chosen for ease of collection. Stable tranfections were selected using neomycin resistance. The cells were grown in serum free conditions and the batches of full length PCPE were purified from volumes of approximately 3 liters of medium. Because this protein carries no tags four different chromatography steps were used to purify full length PCPE. First the medium was passed over lysine Sepharose to adsorb serine proteinases that may degrade PCPE. Material that did not bind the column was passed over a heparin Sepharose column and bound material was eluted with a gradient of sodium chloride (0 to 1M) in 50 mM Tris, pH 7.5. Fractions were analyzed by SDS-PAGE and those containing PCPE were pooled and concentrated. This pool was fractionated on a size exclusion column (BioRad P-30). Fractions were analyzed by SDS-PAGE. Those containing PCPE were mixed with Affi-blue (BioRad) and fractionated by a gradient of 0.5 to 2 M sodium chloride in 50% ethylene glycol. Fractions were analyzed by SDS-PAGE and

reverse zymography. Reverse zymography was required in order to exclude fractions containing TIMP-1. Typical recoveries were approximately 3 mg (i.e. 1 mg from 1 liter of conditioned medium). Purified full length PCPE was subjected to proteolysis with a putative processing enzyme isolated in Task 2. The liberated CT-PCPE was recovered by size exclusion chromatography. This material was found to be active as a metalloproteinase inhibitor by reverse zymography. In collaboration with Dr. Richard Williamson's laboratory this material is currently under investigation by mass spectrometry to determine the functional unit of PCPE. During these experiments, it was observed that if the digestion progressed for a longer time, a smaller non-functional polypeptide was produced. In the future, this material will also be analyzed by mass spectrometry. Comparison of these two fragments will provide information regarding the functional unit of CT-PCPE.

As a part of this Task a polyclonal antibody was to be produced in rabbits. This antibody was required for characterization studies. Purified full length PCPE expressed in HEK-293 cells was used as antigen in a standard immunization protocol to produce a polyclonal antibody. This antibody was produced off site at Covance Research Products, Inc. This antibody has been useful in the characterization of PCPE processing (Task 2) as well as identification of full length and processed PCPE in medium conditioned by breast tumor cell lines (Task 3).

Task 2. To determine the biologically relevant proteinases from breast tumor cells that process intact PCPE to the CT-PCPE metalloproteinase inhibitor. Based on the amino termini of the CT-PCPE identified in brain tumor conditioned medium, five of the six fragments had a signature of serine proteinase cleavage. Moreover, PCPE expressed in a baculovirus system can be processed *in vitro* with plasmin to generate smaller fragments of approximately the same size as CT-PCPE. Some of these small fragments have inhibitory activity as determined by reverse zymography (Mott, et al., 2000). Taken together, this suggests that serine proteinases are most likely the enzymes that process PCPE. We have not observed processing of PCPE in fibroblasts cells. However, we have observed processing of PCPE in a number of cells of non-fibroblastic origin including brain tumor cells, breast tumor cells and other transformed epithelial cells. We have also observed an enzymatic activity that can be recovered from lysine Sepharose chromatography from medium conditioned by brain tumor cells, breast tumor cells and transformed epithelial cells. However, this activity has not been observed or recovered from a normal human fibroblast cell line. Enzyme recovered by lysine Sepharose from medium conditioned by brain tumor, breast tumor and transformed epithelial cells was analyzed by gelatin and casein zymography. A proteinase of apparent molecular weight of 50,000 was observed on both types of zymography from all three cell types. Because this enzyme was not observed in fibroblasts but was observed in several cell lines that process PCPE, this enzyme appeared to be a good candidate for a PCPE processing enzyme. Although this enzymatic activity was present in the aggressive breast tumor cell line MDA MB-231 cells, for ease of purification, the enzymatic activity was isolated from a transformed epithelial cell line using lysine Sepharose and benzamidine affinity chromatography. Medium conditioned by the transformed epithelial cell line was passed over lysine Sepharose and bound material was eluted with 0.2 M ϵ -aminocaproic acid. The recovered protein was dialyzed and passed over a benzamidine column and bound material was eluted with 1 M sodium chloride in 50 mM Tris. The recovered enzyme was determined to be a serine proteinase by treatment with AEBSF (4-(2-aminoethyl)-benzenesulfonyl fluoride), which is a specific and irreversible

inhibitor of serine proteinases. Treatment with AEBSF abolished enzymatic activity on gelatin zymography. Further investigation of this proteinase has suggested that the activity observed at 50,000 is a processed form of a larger latent proteinase. Many proteinases are processed during enzyme activation and such appears to be the case with this particular proteinase. We have made two attempts to identify this proteinase by amino acid sequence analysis. However, no data was obtained from either attempt. This could be due to a blocked amino termini or not enough protein was present to be detected by amino acid sequence analysis. We are currently collecting enough material to make another attempt at sequence analysis.

Although the identity of the proteinase is currently unknown, we have proceeded in the investigation of processing of PCPE by this enzyme. Full length PCPE expressed in HEK-293 cells was purified as described in Task 1. PCPE was mixed with the putative processing enzyme and incubated for 5 or 18 hours. The putative processing enzyme degraded PCPE into fragments that showed inhibitory activity on reverse zymography (Figure 1). The PCPE sample shown in Figure 1 contains a small contamination of TIMP-1. However, a second set of TIMP-1 control experiments was done where purified TIMP-1 was mixed with the processing enzyme using the same conditions as used in Figure 1. No small molecular weight inhibitor was released from the TIMP-1 (data not shown). Moreover, western blot analysis of the samples shown in Figure 1, suggests that the small molecular weight inhibitors appearing in lane 5 are indeed fragments of PCPE (i.e. CT-PCPE) (data not shown).

Results from these experiments suggest that this serine proteinase that is present in brain tumor, breast tumor and transformed epithelial cells can process PCPE to a metalloproteinase inhibitor. This proteinase activity is not observed in normal human fibroblasts and these cells do not process PCPE to the inhibitor CT-PCPE. Taken together these observations suggest that if PCPE is present and this putative processing enzyme is produced then a novel inhibitor is release from cells of non-fibroblastic origin. This may contribute to a difference in the maintenance of ECM proteins surrounding tumor cells and transformed epithelial cells.

Task 3: To determine if increased expression of PCPE and/or CT-PCPE correlates with prognosis and the invasive nature of breast tumor cells. MDA MB-231 cells are a highly invasive cell line that shows signs of epithelial to mesenchymal transition (Gilles and Thompson, 1996; Sommers et al., 1994). Preliminary data of western blot analysis using antibodies to PCPE revealed that fragments of PCPE were present in medium conditioned by MDA MB-231 cells. Using reverse zymography, we have also observed small molecular weight inhibitors in this conditioned medium that do not correspond to TIMPs. Moreover,

we have observed this phenomenon in brain tumor cells and transformed epithelial cells but

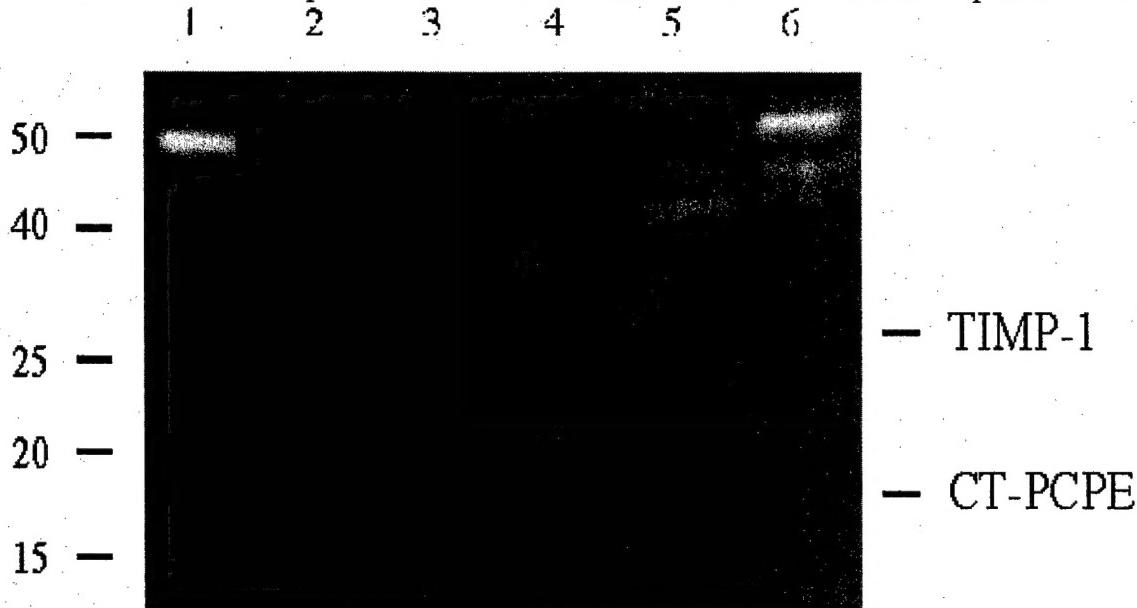


Figure 1. Reverse Zymography of PCPE Digests. Lane 1, Serine proteinase thought to process PCPE at time 0; Lane 2, expressed PCPE at time 0; Lane 3, PCPE and processing enzyme time 0; Lane 4, PCPE and processing enzyme after 5 hours at 37° C; Lane 5, PCPE and processing enzyme after 18 hours at 37° C; Lane 6, processing enzyme after 18 hours 37 °C. Darker areas indicate inhibitor activity. Cleared areas indicate the gelatinolytic activity of the processing enzyme. Full length PCPE stains through the gelatin background in early time points because of the large amount of protein present before digestion.

not normal fibroblasts. Thus, the production of CT-PCPE may be specific to cells that are at a late stage of transformation. To test this hypothesis, we investigated whether different breast tumor cell lines express PCPE and process it to CT-PCPE. Breast tumor cell lines of different degrees of aggressiveness were cultured to confluence. This was also done with a normal human fibroblast cell line (BUD 8). Once confluent, the serum containing growth medium was replaced with serum free maintenance medium. After 48 hours, the conditioned medium was collected and analyzed by reverse zymography and western blot using the polyclonal antibody raised to PCPE in Task 1. As shown in Figure 2, all of the cell lines tested thus far produce TIMP-1. TIMP-2 was also produced in all cell lines. However TIMP-2 activity was not as prominent in MCF 10A (lane 1). In addition to TIMP-1 and TIMP-2, the more aggressive breast tumor cell lines (MDA MB 231 and HS 578T) showed inhibitor activity in the low molecular weight range of 16,500 where CT-PCPE would be expected (lanes 5 and 6).

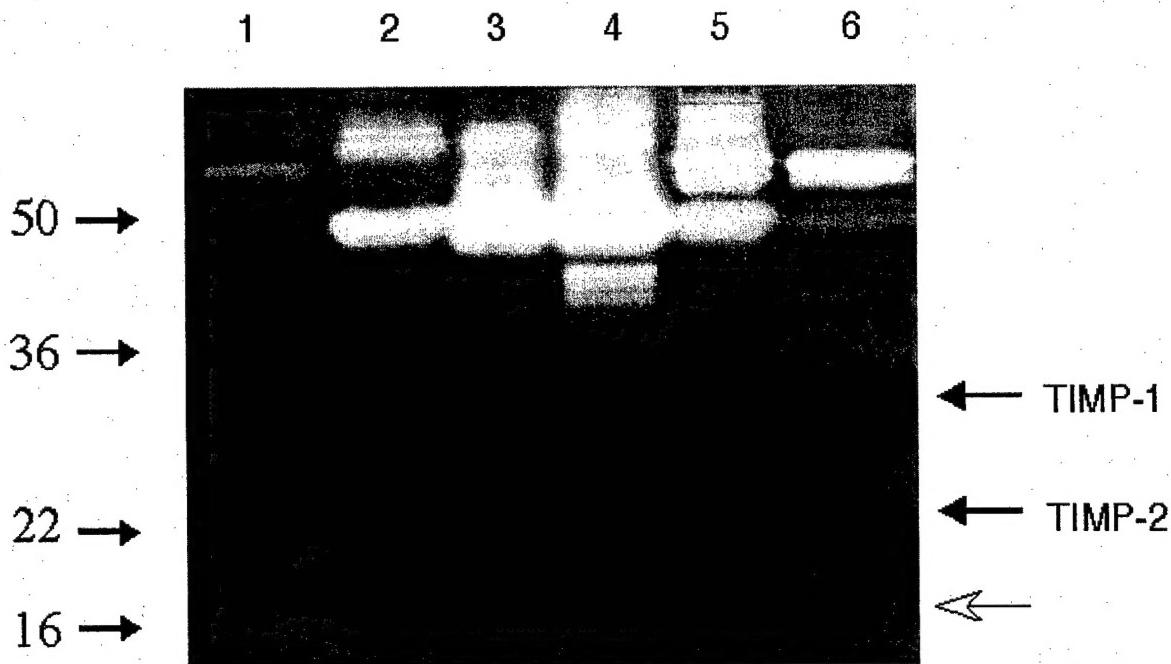


Figure 2. Reverse Zymography of Medium Conditioned by Breast Cell Lines. Lane 1, MCF 10A; Lane 2, MCF 7; Lane 3, MDA MB-468; Lane 4, MDA MB-231; Lane 5, HS 578T; Lane 8, BUD 8 (a normal fibroblast cell line). Positions of TIMP-1 and TIMP-2 are shown at the right. The open arrow shows the position of lower molecular weight inhibitors. This position corresponds to the molecular weight of where CT-PCPE would migrate. Darker areas indicate inhibitor activity and cleared areas indicate gelatinolytic activity of enzymes present in the conditioned medium.

To determine if the activity observed at molecular weight 16,500 in the MDA MB-231 and HS 578T conditioned medium corresponded to CT-PCPE western blot was performed. As shown in Figure 3, except for MCF 10A, all cell lines tested thus far produce full length PCPE. Furthermore, the more aggressive cell lines of MDA MB-231 and HS 578T processed PCPE to smaller fragments. These smaller fragments of CT-PCPE observed in MDA MB-231 may account for the inhibitor activity observed in the reverse zymography. To verify this, future studies will be required. The important observations made from these analyses are (1) a "normal" breast epithelial cell line did not produce PCPE; (2) although all the breast tumor cell lines analyzed produced full length PCPE only the more aggressive lines processed PCPE; (3) a normal fibroblast cell line produced full length PCPE but did not process PCPE. These observations suggest that as breast epithelial cells become more aggressive and proceed through epithelial to mesenchymal transition, PCPE is produced followed by the production of a proteinase that can process PCPE into fragments. Potentially the C-terminal fragment CT-PCPE can act as a metalloproteinase inhibitor. On the other hand, as demonstrated by a normal human fibroblast cell line, fibroblasts produce PCPE but they do not process PCPE. Therefore, as breast tumor cells become more aggressive, they may alter the expression of proteinases and inhibitor that are required to maintain ECM proteins. This may lead to an environment that allows tumor cell invasion and/or fibrosis.

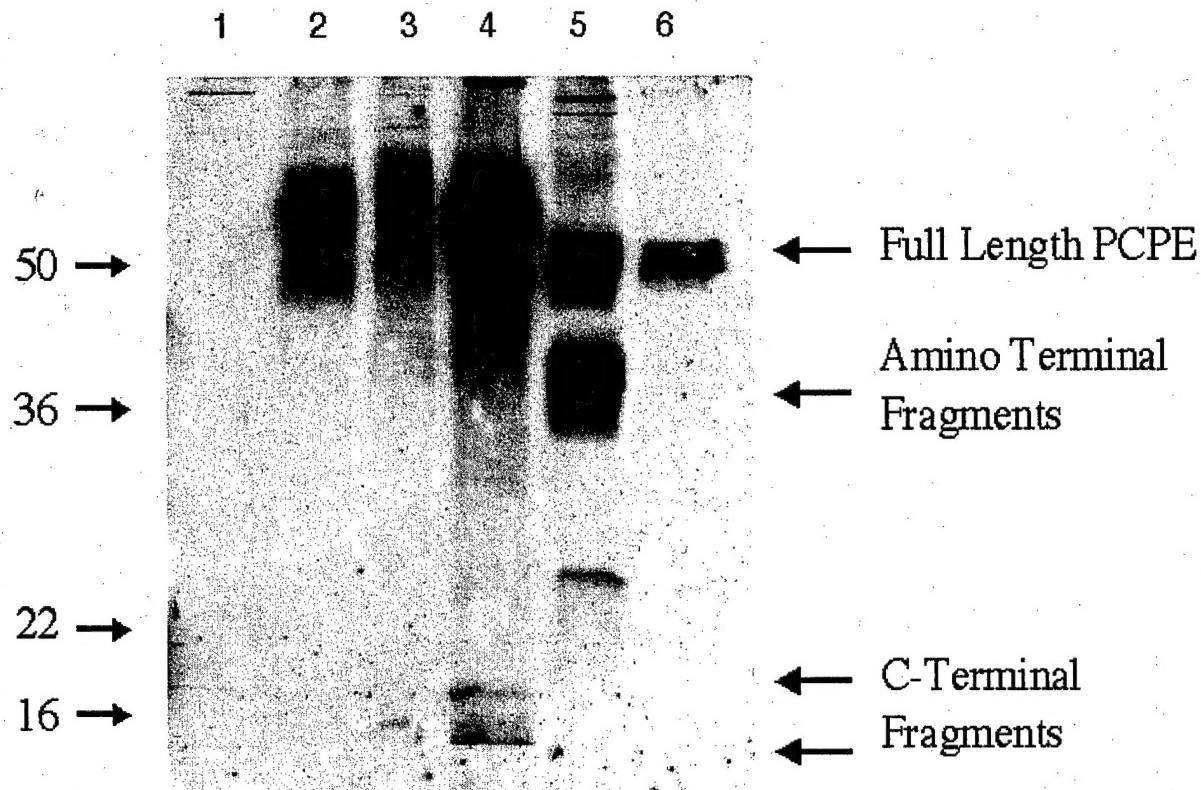


Figure 3. Western Blot Analysis of Medium Conditioned by Breast Cell Lines. Lane 1, MCF 10A; Lane 2, MCF 7; Lane 3, MDA MB-468; Lane 4, MDA MB-231; Lane 5, HS 578T; Lane 8, BUD 8 (a normal fibroblast cell line). Positions of full length PCPE as well as N-terminal and C-terminal fragments of PCPE are shown at the right. Larger molecular weight bands that have reacted with the PCPE antibodies may represent different glycosylation states of PCPE.

Key Research Accomplishments

- 1 We have scaled up expression of PCPE in order to purify enough material to use as an antigen for the production of a polyclonal antibody in rabbits. This antibody has been very useful in the characterization of PCPE and its fragments from *in vitro* experiments and for probing medium conditioned by cell lines. Also we can generate enough purified full length PCPE in order to characterize processing of the protein by *in vitro* assays.
- 2 Isolated a putative PCPE processing enzyme. We have shown that this serine proteinase can process full length PCPE *in vitro* and liberate fragments that have inhibitor activity on reverse zymography. We are currently trying to obtain enough of this proteinase to identify by amino acid sequence analysis.
- 3 Using reverse zymography and western blot, we have observed that breast tumor cell lines express full length PCPE and that the more aggressive tumor cell lines also process full length PCPE. Suggesting that the more aggressive cells produce a PCPE processing enzyme as well as PCPE. This is in contrast to a normal breast epithelial cell line (MCF 10A), which does not produce PCPE. Additionally, although normal fibroblasts produce PCPE, they do not process the protein. This suggests that they do not produce a PCPE processing enzyme. Thus, production of PCPE followed by processing PCPE to a metalloproteinase inhibitor may be markers of progression of breast tumor cells from a less aggressive to a more aggressive phenotype.

Reportable Outcomes

Welm, B., Mott, J., and Werb, Z (2002) Developmental Biology: Vasculogenesis is a Wreck Without RECK. Curr. Biol. 12:R209-211.

Wiseman, B.S., Sternlicht, M.D., Lund, L.K. Alexander, C.M., Mott, J., Bissell, M.J., Soloway, P. Shigeyoshi, I., and Werb, Z. (2002) Site-specific Positive and Negative Activities of MMP-2 and MMP-3 Orchestrate Mammary Gland Branching Morphogenesis. Devel. Cell Submitted.

Mott, J.D. and Banda, M.J. (2002) Procollagen C-terminal Proteinase enhancer in Breast Tumor Cell Lines. DOD Era of Hope Meeting, Orlando, FL.

Mott, J.D., Bhatnagar, S. and Banda, M.J. (2002) A Serine Proteinase in Medium Condition by the Epithelial Cell line HEK 293 Processes Procollagen C-terminal Proteinase Enhancer Releasing a Metalloproteinase Inhibitor. Abstract number 2746. Annual Meeting American Society for Cell Biology, San Francisco, CA.

Conclusions

The non-TIMP metalloproteinase inhibitor CT-PCPE has not been observed in the normal human fibroblast cell line Hs 27. The small molecular weight inhibitor CT-PCPE has been observed in brain tumor cell lines, breast tumor cell lines and a transformed epithelial cell line. Taken together, these observations suggest that CT-PCPE production may only occur in transformed epithelial cells. The biology of MMP and their classical inhibitors, TIMP, has typically been studied in fibroblasts, other stromal cells, or vascular endothelial cells. However, one of the most striking observations from our research is that the non-TIMP metalloproteinase inhibitor we have designated CT-PCPE appears to be present exclusively in transformed epithelial cells and cells of non-stromal origin. Thus, the production of CT-PCPE from PCPE may be a marker for transformed and potentially invasive cells. We will be continuing to examine a number of breast tumor epithelial cell lines both invasive and non-invasive to determine if CT-PCPE is a marker for the invasive nature of breast epithelial cells. Information gathered from this data may be useful in designing assays to screen for CT-PCPE. This information may be helpful in deciding the course of treatment for specific breast tumors.

In addition to evaluating the correlation of CT-PCPE with the aggressive nature of breast tumor cells, we are continuing to establish the importance of CT-PCPE as a metalloproteinase inhibitor. In an effort to understand mechanisms by which CT-PCPE inhibits MMP, the structure of CT-PCPE is being determined. We have made progress in determining the structure of CT-PCPE through our collaboration with Dr. Richard Williamson. Additionally, we have identified and isolated a serine proteinase that can process PCPE *in vitro*. We are in the process of collecting enough of this proteinase to identify by amino acid sequence analysis. Understanding mechanisms that modulate MMP activity is important for the design of compounds that can specifically inhibit MMP. Modulation of MMP activity may be an important avenue for controlling breast tumor cell invasion and metastasis.

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